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(57) Abstract

The present invention relates to a purified protein that antagonizes the ability of chemokines (CC and CXC) to attract leukocytes (monocytes, lymphocytes, and neutrophils), and it's use as an anti-inflammatory and antiviral agent. A method for treating a chemokine related immunopathological disorder in a subject by administering a therapeutically effective amount of an anti-inflammatory protein, MCVCC, encoded by the molluscum contagiosum virus (MCV) gene MC148 (genome location from about base-pair 166,992 to base-pair 167,303 of MCV, or a biologically active fragment thereof) is provided. A method for treating a subject having or at risk of having an HIV infection or disorder by administering a therapeutically effective amount of this anti-inflammatory protein is also provided. A pharmaceutical composition containing at least one dose of an anti-inflammatory protein having the amino acid sequence of a protein encoded by the MCV genome from about base-pair 166,992 to base-pair 167,303, or a biologically active fragment thereof, in a therapeutically acceptable carrier, is also provided.

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BROAD SPECTRUM CHEMOKINE ANTAGONIST AND USES THEREOF

FIELD OF THE INVENTION

This invention relates generally to the field of immunology, and more specifically to a chemokine antagonist and its use as an anti-inflammatory and antiviral agent.

BACKGROUND OF THE INVENTION

It is becoming increasingly clear that viruses which replicate within cells of higher-order vertebrates must have evolved to specifically avoid the host immune system (Gooding, L., Cell, 91:5-7, 1992; Marrack, P. and Kappler, J., Cell, 76:323-332, 1994; Smith, G., Trends in Micro., 82:80-88, 1994). In fact, virus survival is dependent upon strategies which can evade, suppress, counteract, or otherwise confound the myriad of host responses to a foreign invader. The larger DNA viruses (*i.e.*, the adenoviruses, herpesviruses, iridoviruses and poxviruses) specifically encode proteins that function to protect the virus from immune recognition and/or clearance by the infected host. Such "subversive" viral proteins are now providing information concerning the functional operations of the immune system, and it is likely that many more discoveries of new members of this growing family will be identified in the future.

In the 1980's the term "virokine" was proposed to describe virus-encoded proteins secreted from infected cells which function by mimicking extracellular signaling molecules such as cytokines or other secreted regulators important for the host immune repertoire (Kotwal, G. and Moss, B., Nature, 335:176-178, 1988). Later, in the 1990's the term "viroceptor" was introduced to account for the observation that some virus encoded proteins that mimic important cellular receptors and function by diverting host cytokines away from their normal receptors, thus interrupting the immune circuitry at its earliest stages (Upton, et al., Virology, 184:370, 1991; Schreiber and McFadden, Virology, 204:692-705, 1994).

The poxvirus family of large-double stranded DNA viruses is composed of numerous species that infect vertebrates and insects, but only two of which are specific for humans: (1) variola virus, which was responsible for smallpox, and (2) molluscum contagiosum virus (MCV), which produces papules that may persist in the skin of young children and sexually active adults for months before spontaneously regressing. MCV is also responsible for an extensive and essentially untreatable opportunistic disease that occurs in individuals with AIDS (Gottlieb, S.L., and Myskowski, P.L., 1994, Int. J. Dermatol. 33:463). Infection with MCV elicits a weak immune response, but little is known of the basis for the MCV/host interaction. The MCV major subtype 1 DNA has been sequenced, and predicted to encode 163 proteins, 103 of which have homologs in the smallpox virus, and 59 that encode unique proteins, including MC148 (Senkevich, T.G., et al., 1996, Science 263:813-816). The MC148 gene product has been shown to inhibit the growth of hematopoietic progenitor cells *in vitro*, and has been postulated to be involved in immune evasion (Krathwohl., M.D., et al., 1997, Proc. Natl. Acad. Sci. 94:9875-9880).

Immunomodulatory proteins include chemotactic cytokines called "chemokines." Chemokines are small inducible proteins that are related by amino acid homology, chromosome location, and structural similarities. The chemokines are chemoattractants for leukocytes, especially neutrophils, basophils, monocytes, and T cells. The structure of the chemokines includes the presence of four position-invariant cysteine residues in their primary amino acid sequence that form two disulfide bonds.

Certain chemokines, known as alpha or CXC chemokines, have a Cys-X-Cys triplet as the first two cysteines (X can be any amino acid other than cysteine), and include the human-derived proteins interleukin-8 (IL-8), GRO-α (also called melanoma-growth stimulating activity (MGSA/GRO), MIP-2α (also known as GRO-β), MIP-2β (also known as GRO-γ), neutrophil activating peptide-2 (NAP-2), platelet factor 4 (PF4), gamma interferon inducible protein 10 (γIP-10), Epithelial derived Neutrophil Activating protein (78 amino acids in length)(ENA-78), β-thromboglobulin (βTG), connective

tissue-activating peptide-III (CTAP-III), and platelet basic protein (PBP). The alpha chemokines are potent chemoattractants and except for PF4 and γIP-10, activate neutrophils.

Other chemokines, known as beta or CC chemokines, have a Cys-Cys pair as the first two cysteines, and include macrophage inflammatory protein-1 alpha (MIP-1α), MIP-1β, macrophage chemotactic and activating factor (MCAF, also known as monocyte chemo-attractant protein-1 (MCP-1)), MCP-3, and Regulated on Activation, Normal T-cell Expressed and Secreted protein (RANTES). The beta chemokines are potent chemoattractants for a variety of blood cell components, including monocytes, eosinophils, and T-lymphocytes, but not neutrophils. Recently, a third group of chemokines, the "C" group, was designated by the discovery of a new protein called lymphotactin (Kelner et al., 1994, Science 266:1395-1399). Chemokines have been shown to regulate proliferation and/or differentiation of hematopoietic stem and progenitor cells *in vitro* and *in vivo*. The chemokine family is also believed to be critically important in the infiltration of monocytes and lymphocytes into sites of inflammation.

SUMMARY OF THE INVENTION

The present invention relates to a purified protein that antagonizes the ability of chemokines (CC and CXC) to attract leukocytes (monocytes, lymphocytes, and neutrophils), and it's use as an anti-inflammatory and antiviral agent.

The present invention provides a method for treating a chemokine related immunopathological disorder in a subject by administering a therapeutically effective amount of an anti-inflammatory protein, MCVCC, encoded by the molluscum contagiosum virus (MCV) from about base-pair 166,992 to base-pair 167,303, or a biologically active fragment thereof. A method for treating a subject having or at risk of having an HIV infection or disorder by administering a therapeutically effective amount of an anti-inflammatory protein encoded by MCV from about nucleotide 166,992

to nucleotide 167,303, or a biologically active fragment thereof, is also provided. A pharmaceutical composition containing at least one dose of an anti-inflammatory protein having the amino acid sequence of a protein encoded by the MCV genome from about base-pair 166,992 to about base-pair 167,303, or a biologically active fragment thereof, in a therapeutically acceptable carrier, is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the steps of MCVCC purification. Lane 1 is clarified supernatant, lane 2 is pooled 280-370 mM NaCl fractions from the heparin column, and lane 3 is the diluted material from 145-160 ml elution volumes from the G50 column. Lane four represents the purified material from the final cation exchange column. Lane 5 contains molecular weight markers.

Figure 2 shows representative chemotaxis inhibition experiments. Figure 2A shows the ability of MCVCC to inhibit chemotaxis of monocytes to MCP-1 in a dose dependent fashion. Heat treated (ΔMCVCC) MCVCC does not have this effect, nor does an excess of IL-8. Specific inhibition of monocyte chemotaxis to MCP-3, MIP-1a, RANTES, I-309, and SDF-1 by MCVCC is shown in Figure 2B-Figure 2F. Figure 2G depicts the inability of MCVCC to inhibit monocyte chemotaxis to fMLP. Figure 2H and Figure 2I demonstrate the unique ability of MCVCC to inhibit chemotaxis of lymphocytes and neutrophils to the CXC chemokines SDF-1 and IL-8, respectively.

Figure 3 shows the ability of MCVCC to act as a chemokine antagonist, as evidenced by inhibition of chemokine induced intracellular calcium release. Figure 3A shows in primary monocytes, that where 50 nM MCVCC partially inhibits, and 120 nM MCVCC completely inhibits the calcium release to 1 nM MCP-1. Figure 3B shows a similar effect in specific CCR2b chemokine receptor transfected HEK293 cells. Figure 3C shows that 1.2 nM MCVCC partially, and 12 nM MCVCC completely inhibits intracellular calcium release of CCR8 transfected murine preB cells to 0.5 nM I-309.

Figure 4A shows a graph illustrating the ability of MCVCC to compete with MCP-1 for binding to CCR2b transfected HEK293 cells. The IC_{50} of MCP-1 and MCVCC are similar.

Figure 4B shows a graph illustrating the ability of MCVCC competes with I-309 for binding to CCR8 transfected murine preB cells. The IC_{50} of I-309 and MCVCC are similar.

Figure 4C shows a bar graph illustrating the ability of MCVCC to inhibit fusion of HIV strain 89.6 envelope with cells expressing CD4 and CCR5.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the receptor" includes reference to one or more receptors and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the cell lines, chemokines, and methodologies which are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application.

Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The present invention provides a new method of use of the MCV chemokine homolog, MCVCC, as a chemokine antagonist for CC and CXC chemokines. MCVCC is useful as an anti-inflammatory agent, or for treating HIV infection. While not wanting to be bound by a particular theory, it is believed that the MCVCC polypeptide acts broadly as a chemokine antagonist or as an anti-viral agent by binding to or blocking the chemokine receptor, rather than binding to the chemokine or virus, respectively, itself. Thus, the MCVCC polypeptide may be useful as an antagonist for any chemokine.

A "chemokine" is a class of cytokines which are responsible for leukocyte chemotaxis. The α class of chemokines is designated CXC (where X is any amino acid), and includes interleukin-8 (Il-8), connective tissue activating protein III (CTAP-III), melanocyte growth stimulatory activity (MGSA) gro/MGSA, inducible protein (IP-10), neutrophil activating peptide 2 (NAP2), β-thromboglobulin and epithelial-derived neutrophil attractant-78 (ENA-78); and the β class, designated C-C, which includes I-309 T-cell activation gene-3 (TCA-3), monocyte chemotactic proteins (MCP-1, 2, and 3), macrophage inflammatory proteins (MIP-1α and β), and regulated on activation, normal T expressed and secreted protein (RANTES). Chemokines activate leukocytes by binding to selective, seven-transmembrane-domain, G protein-coupled receptors present on the plasma membrane. Nine CC receptors (designated CCR1,2a,2b, and CCR3-9) and four CXC receptors (designated CXCR1, CXCR2, CXCR3, and CXCR4) have been described, and additional putative "orphan" chemokine receptors have been found by searching sequence databases for homology to known chemokine receptors (Murphy, P.M., 1996, "Chemokine receptors: structure, function, and role in microbial pathogenesis," Cytokine Growth Factor Review 7:47-64; Roos *et al.*, 1997, "Identification of CCR8, the receptor for the human CC chemokine I-309", J. Biol Chem. 272:17251-4; Tiffany *et al.*, "Identification of CCR8: a human monocyte and thymus receptor for the CC chemokine I-309", J. Exp. Med. 186:165-70). Most of the receptors

identified so far bind multiple chemokines; most chemokines tested so far bind to two or more receptor subtypes. To date, no CC chemokine has been shown to interact with a CXC receptor and no CXC chemokine with a CC receptor. Examples of chemokines that bind to some of the chemokine receptors are listed in Table 1. In addition to their suspected beneficial role in host defense and tissue repair, several chemokine receptors (CCR2, CCR3, CCR5 and CXCR4) are exploited pathologically by HIV-1, acting together with CD4 as cell entry coreceptors *in vitro* (G. Alkhatib et al., Science (1996) 272:1955). The HIV-1 strain specificity of the coreceptors is complex.

Table 1

CXC Receptors and Their Ligands

Receptor	Ligands
CXCR1	IL-8
CXCR2	IL-8, Gro-α/MGSA, NAP-2, CTAP-III, ENA-78, PF-4
CXCR3	IP-10, MIG
CXCR4	SDF-1
CCR1	MIP-1α, MIP-1β, MCP-3, RANTES, MCP-1
CCR-2a,-2b	MCP-1, MCP-3
CCR-3	Eotaxin, RANTES, MCP-3, MCP-4
CCR-4	MIP-1α, RANTES, TARC
CCR-5	MIP-1α, MIP-1β, RANTES

Other chemokines can be detected by methods commonly used in the art. For example, a molecule may be tested using the Boyden chamber, which is the preferred microchemotaxis assay system for *in vitro* investigation of chemoattractant substances. A series of wells is formed into a plexiglass block, each well consisting of two chambers, upper and lower, which are separated by any one of several types of porous filters, such as nitrocellulose and polycarbonate, for example. The cell of interest, for example peripheral blood mononuclear cells (PBMC), are added to the top chamber of each well and the test substance, *e.g.*, the chemoattractant, is added to the bottom chamber. If the cells in the top chamber are attracted to the substance in the bottom chamber, they will migrate along the theoretical concentration gradient which exists in solution and move through the pores of the filter and adhere to the bottom side of that filter.

An "antagonist"is any chemical substance capable of reducing the biological activity of another chemical substance. The term "antagonist" includes, but is not limited to molecules with significant similarity to a second molecule to compete with the molecule for binding sites on a third molecule. The term "antagonist" also includes, but is not limited to molecules which bind to a second molecule to reduce the binding of the second molecule to a third molecule.

The MCVCC polypeptide useful in the methods of the invention is encoded by the MC148 gene, located from about nucleotide 166,992 to nucleotide 167,303 of MCV (Senkevich, T.G., *supra*) (SEQ ID NO:1). Two major subtypes of MCV, MCV1 and MCV2, are also known. In addition, naturally occurring variants or polymorphisms of MCV1 and MCV2, as indicated by heterogeneity of restriction endonuclease cleavage of MCV1 and MCV2 DNA, are also known to exist (Porter, C.C., et al., 1987, Epidemiol. Infect. 99:563-7). The MCVCC useful with in methods of the invention include MCVCC encoded by MCV1, MCV2, or naturally occurring variants of either MCV1 and MCV2.

The invention includes the functional polypeptide, MCVCC (SEQ ID NO:2), and biologically functional fragments thereof. As used herein, the term "functional polypeptide" refers to a polypeptide which possesses a biological function or activity which is identified through a defined functional assay and which is associated with a particular biologic, morphologic, or phenotypic response. Functional fragments of the MCVCC polypeptide include fragments of MCVCC that have an activity of MCVCC (*e.g.*, CC chemokine antagonist, CXC chemokine antagonist, or inhibiting cell membrane fusion mediated by HIV-1 envelope protein). Smaller or larger peptides containing the biological activity of MCVCC are included in the invention. Such peptides can be assayed for activity as a chemokine antagonist, for the ability to inhibit cell membrane fusion mediated by the interaction of the HIV-1 envelope protein, for the ability to inhibit monocyte migration, or any other function of MCVCC, using methods commonly known to those of skill in the art, including methods described in the EXAMPLES herein. For

example, a Boyden chamber is useful for identifying chemokines as described herein. The biological functional MCVCC can vary from a peptide fragment as small as an epitope to which an antibody molecule can bind to a large polypeptide which is capable of functioning as a chemokine antagonist. A "functional polynucleotide" denotes a polynucleotide which encodes a functional polypeptide as described herein.

Minor modifications of the MCVCC primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the MCVCC polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications or that are naturally occurring are included herein as long as the biological activity of MCVCC is retained (*e.g.*, CC chemokine antagonist, CXC chemokine antagonist, or inhibiting cell membrane fusion mediated by HIV-1 envelope protein). Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its activity. This can lead to the development of a smaller or variant active molecule which would have broader utility. For example, it is possible to remove amino or carboxy terminal amino acids which may not be required for MCVCC activity, or may even provide increased activity over wild-type MCVCC.

The MCVCC polypeptide useful with the methods of the invention also include conservative variations of the polypeptide sequence. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted

polypeptide also immunoreact with the unsubstituted polypeptide. The invention includes naturally occurring variants, as well as purposely designed variants, which exhibit anti-inflammatory activity, or another biological activity of MCVCC.

The invention provides a method for treating an immunopathological disorder in a subject. The method includes administering to the subject a therapeutically effective amount of an MCVCC anti-inflammatory protein. The term "anti-inflammatory" refers to reduction, suppression, or inhibition, of an inflammatory response.

An immunopathological disorder treated by the method of the invention may be associated with production of chemokines and resultant accumulation of reactive leukocytes at afflicted tissues. The method comprises administering to the subject a therapeutically effective amount of MCVCC. The term "immunopathological disorder" refers to any disease which involves the immune response or immunity in general. Examples of immunopathological diseases are microbial infection, malignancy and metastasis, asthma, coronary restenosis, autoimmune diseases, cirrhosis, endotoxemia, rheumatoid arthritis, atherosclerosis, psoriasis, inflammatory bowel disease, interstitial cystitis and reperfusion injury, amongst others. Immunopathological disorders which can be treated by the method of the invention also include acquired immunodeficiency disorder (AIDS), or ARC, toxic shock syndrome, allograft rejection, atherosclerotic plaque growth, ultraviolet and radiation responses, and disorders associated with the activation of T cells, B cells, macrophages, and other inflammatory leukocytes during the immune response and the acute phase response and disorders associated with advanced cancer such as tumor necrosis factor-mediated cachexia. The invention provides a method of treating or ameliorating an immunopathological disorder including endotoxemia or septic shock (sepsis), or one or more of the symptoms of sepsis comprising administering to a subject displaying symptoms of sepsis or at risk for developing sepsis, a therapeutically effective amount of MCVCC.

"Therapeutically effective" as used herein, refers to that amount of MCVCC that is of sufficient quantity to ameliorate the cause of the immunopathological disorder. The term "ameliorate" refers to a decrease or lessening of the symptoms of the disorder being treated. The subject of the invention is preferably a human, however, it can be envisioned that any animal with an immunopathological disorder can be treated by the method of the invention, for example, a SCID mouse grafted with human bone marrow (humanized SCID).

A patient who exhibits the symptoms of an immunopathological disorder may be treated with an antibiotic or antiviral agent in addition to the treatment with MCVCC. Antibiotics include an aminoglycoside, such as gentamycin or a beta-lactam such as penicillin, or cephalosporin. Therefore, a therapeutic method of the invention includes administering a therapeutically effective amount of MCVCC substantially simultaneously with administration of a bactericidal amount of an antibiotic or sufficient amount of an anti-viral compound.

The term "bactericidal amount" as used herein refers to an amount sufficient to achieve a bacteria-killing blood concentration in the patient receiving the treatment. The bactericidal amount of antibiotic generally recognized as safe for administration to a human is well known in the art, and as is known in the art, varies with the specific antibiotic and the type of bacterial infection being treated. Preferably, administration of MCVCC occurs within about 48 hours and preferably within about 2-8 hours, and most preferably, substantially concurrently with administration of the antibiotic.

Administration of a MCVCC in the method of the invention may also be used for ameliorating post-reperfusion injury. When treating arterial thrombosis, induction of reperfusion by clot lysing agents such as tissue plasminogen activator (t-PA) is often associated with tissue damage. Such tissue damage is thought to be mediated at least in part by leukocytes including, but not limited to, polymorphonuclear leukocytes (PMN). Therefore administration of the MCVCC would block leukocyte or PMN-endothelial

interactions, and thereby diminish or prevent post-reperfusion injury. Administration of MCVCC is also useful for prevention of new onset and recurrent atherosclerotic plaque growth after arterial injury. Restenosis and new growth of plaque is believed to be exacerbated by the local inflammatory response to the internal layer of the artery wall.

The method of the invention is also useful for treatment of inflammation due to allergic or autoimmune disorders. Examples of allergic disorders include allergic rhinitis, asthma, atopic dermatitis, and food allergies. Examples of autoimmune disorders, where the immune system attacks the host's own tissues, include, but are not limited to, type 1 insulin-dependent diabetes mellitus, inflammatory bowel disease, dermatitis, meningitis, thrombotic thrombocytopenic purpura, Sjögren's syndrome, encephalitis, uveitis, leukocyte adhesion deficiency, rheumatoid and other forms of immune arthritis, rheumatic fever, Reiter's syndrome, psoriatic arthritis, progressive systemic sclerosis, primary biliary cirrhosis, pemphigus, pemphigoid, necrotizing vasculitis, myasthenia gravis, multiple sclerosis, lupus erythematosus, polymyositis, sarcoidosis, granulomatosis, vasculitis, pernicious anemia, CNS inflammatory disorder, antigen-antibody complex mediated diseases, autoimmune hemolytic anemia, Hashimoto's thyroiditis, Graves disease, habitual spontaneous abortions, Reynaud's syndrome, glomerulonephritis, dermatomyositis, chronic active hepatitis, celiac disease, autoimmune complications of AIDS, atrophic gastritis, ankylosing spondylitis and Addison's disease.

The method is also useful in treating non-malignant or immunological-related cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Behcet's syndrome, acute respiratory distress syndrome (ARDS), ischemic heart disease, atherosclerosis, post-dialysis syndrome, leukemia, acquired immune deficiency syndrome, septic shock and other types of acute inflammation, and lipid histiocytosis. Essentially, any disorder which is etiologically linked to the pro-inflammatory process and cellular infiltration due to chemokine production (*e.g.*, induction of IL-8, MIP-1α or β expression) would be considered susceptible to treatment.

The method of the invention is also useful for the treatment of microbial infections. Many microbes, such as bacteria, rickettsia, various parasites, and viruses, bind to vascular endothelium and leukocytes, and induce an inflammatory reaction resulting in production of interleukins for example. Thus, the MCVCC used in the method of the invention may be administered to a patient to prevent inflammation associated with such infections.

The present invention also provides a method and compositions for treatment of a subject having or at risk of having an human HIV associated disorder. HIV associated disorders include, for example, without limitation: AIDS, Kaposi's sarcoma, and Hodgkin's B-cell lymphoma.

The dosage ranges for the administration of the MCVCC of the invention are those large enough to produce the desired effect in which the symptoms of the immune response show some degree of suppression. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about 10 pg to 100 μg per dosage, in one or more dose administrations daily, for one or several days.

The MCVCC is administered by any suitable means, including parenteral, subcutaneous, intrapulmonary, intraarterial, intrarectal, intramuscular, and intranasal administration. Parenteral infusions include intramuscular, intravenous, intraarterial, or intraperitoneal administration. MCVCC may also be administered transdermally in the form of a slow-release subcutaneous implant for example, or orally in the form of capsules, powders or granules. MCVCC can also be administered by inhalation. For example, when used therapeutically for treatment of an inflammatory disorder of the lungs, a preferred route of administration would be by a pulmonary aerosol.

Pharmaceutically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients include water, saline, dextrose, glycerol and ethanol, or combinations thereof. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the MCVCC of the invention, the medicament being used for therapy of an undesirable immune response/inflammatory reaction wherein the immune response results in production of chemokines which bind to either a CC or a CXC chemokine receptor.

The invention provides a pharmaceutical composition comprising at least one dose of an immunotherapeutically effective amount of an anti-inflammatory protein having the amino acid sequence of a protein encoded by the MCV genome from about base-pair 166,992 to base-pair 167,303, and having the biological function of MCVCC, in a pharmacological carrier.

The invention provides any pharmaceutical preparations and compositions containing the MCVCC of the invention for use in the method of the invention. The form will vary

depending upon the route of administration. For example, compositions for injection can be provided in the form of an ampule, each containing a unit dose amount, or in the form of a container containing multiple doses.

MCVCC can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. These include the acid addition salts which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acid, or organic acids such as acetic, oxalic, tartaric and the like. Salts also include those formed from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and organic bases such as isopropylamine, trimethylamine, histidine, procaine and the like.

Controlled delivery may be achieved by selecting appropriate macromolecules, for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate, or lactide/glycolide copolymers. The rate of release of the MCVCC may be controlled by altering the concentration of the macromolecule.

Another method for controlling the duration of action comprises incorporating the MCVCC into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, polylactide/glycolide copolymers, or ethylenevinylacetate copolymers. Alternatively, it is possible to entrap MCVCC in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly(methylmethacrolate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLE 1:

EXPRESSION AND PURIFICATION OF MCVCC

Proteins encoded by MCV cannot be obtained in quantity from natural sources since the virus has not been grown in the laboratory or in experimental animals. PCR was used to amplify the MC148 open reading frame with the sequence preceding it optimized for translation in mammalian cells (Kozak, M., 1992, "Regulation of translation in eukaryotic systems," Ann. Rev. Cell Biol. 8:197-225) and with *Eco*RI and *Nco*I restriction sites to facilitate cloning the DNA in the plasmid vector pRB21 (Blasco *et al.*, 1995 "Selection of recombinant vaccinia viruses on the basis of plaque formation" Gene 158:157-162) The oligonucleotides 5'GCGGAATTCGCCACCATGGGGAGGGGCGGAGACGTCTTCGCGAG3' (SEQ ID NO:3) and 5'GCGGCCATGGTTACAGAGACTCGCACCCGGACCATATC3' (SEQ ID NO:4) were used for the PCR and DNA from four isolates of molluscum contagiosum served as templates. The cloned DNA was sequenced to ensure that the MC148 gene was intact. Two species of the protein were detected, one with a mature amino terminal sequence LARRKCC, the other with the mature amino terminal sequence LAKRKCC. Each plasmid was then used to construct a recombinant vaccinia virus (vIDA-2 and vIDA-1), respectively, to express the gene product (Blasco *et al.*, *supra*). This procedure used an optimal sequence for translation.

Expression of a secreted form of the MCVCC by mammalian cells infected with the recombinant virus was demonstrated by polyacrylamide gel electrophoresis (Fig. 1). The predicted 9.8 kD protein was detected by silver staining and immunoblotting using polyclonal antisera directed against a 35 amino acid synthetic peptide. The amount of

MCVCC detected in the medium of vIDA-2 infected cells was about threefold greater than that previously achieved with suboptimal translation initiation sequences. The high expression facilitated the purification of MCVCC.

A preferred method of purification of MCVCC involved the infection of seventy to ninety 150 cm^2 flasks containing monolayers of BS-C-1 or HeLa cells in Optimem (Gibco/BRL) with 10 infectious units per cell of vIDA-2. After 30 to 35 hours, the medium of the cells was harvested. Any suspended cells were removed by centrifugation at 1,000 x g for 10 minutes. The supernatant was adjusted to contain 0.1% Triton X-100 and clarified by centrifugation at 10,000 X g for 30 minutes. The high speed supernatant was then applied to a HiTrap heparin column (Pharmacia) that had been equilibrated with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl. A linear gradient of 100 mM to 600 mM NaCl in 50 mM Tris-HCl (pH 7.4) was used to elute material from the column. Fractions between 280 mM and 370 mM NaCl containing MCVCC were pooled and applied to a 200 ml G50 gel filtration column that had been equilibrated with 20 mM sodium phosphate (pH 7.4), 200 mM NaCl. Elution volumes between 145 and 160 ml contained the relatively pure 10 kD MC148 protein. Calibration of the G-50 column with ovalbumin (43 kD), ribonuclease A (13.5 kD), and insulin (6.5 kD) demonstrated that MCVCC eluted between ribonuclease A and insulin, consistent with it being largely monomeric. The fractions containing MCVCC were pooled and diluted threefold with 20 mM sodium phosphate (pH 7.4) and applied to the final HiTrap SP column equilibrated with 20 mM sodium phosphate (pH 7.4), 50 mM NaCl. Material was eluted from the cation exchange column with a linear, 50 to 500 mM NaCl gradient. Polyacrylamide gel electrophoresis revealed a single 10 kD protein species, detected by silver staining, that eluted between 270 and 370 mM NaCl. The protein reacted with polyclonal MC148 antipeptide serum but not with preimmune serum. A silver stained polyacrylamide gel indicating the purity at each step of the purification is shown in Fig 1. The NH2-terminal sequence of purified MCVCC confirmed the predicted signal peptide cleavage site (Senkevich, T.G., et al., 1996, *supra*), and in protein purified from vIDA-1 detected a mature amino terminal sequence LAKRK. Most experiments utilized

the MCVCC protein obtained from vIDA-2 infected cells with the LARRK amino terminal amino acid sequence; limited experimental data with the LAKRK amino terminal amino acid sequence protein showed similar results.

EXAMPLE 2:

METHODS FOR MEASURING BROAD SPECTRUM CHEMOKINE ANTAGONISTIC ACTIVITY

Several assays were used to demonstrate chemokine antagonistic activity. One preferred method was to measure the effect of MCVCC on the ability of chemokines to attract leukocytes since this is most directly related to inflammation. Inhibition by MCVCC of chemotaxis of freshly elutriated monocytes from random donors was analyzed in a 48 well microwell Boyden chamber (Neuroprobe) with polyvinylpyrrolidone (PVP) coated polycarbonate filters (pore size 5 μm) and a 40 minute incubation at 37°C. Procedures well known in the art were used for preparation and analysis Leonard, E.J., et al., 1995, "Measurement of α and β chemokines," In: Curr. Protocols Immunol., vol. 1, John Wiley & Sons, pp.6.12.1-6.12.28). Dilutions were made in RPMI 1640 (Quality Biological, Inc.) containing 0.5% BSA and 25 mM Hepes pH 7.4. All experiments were performed at least three times. Migrated cells were counted in five 40X fields. Chemotaxis experiments with I-309 used the method of Miller and Krangel (1992, Proc Natl Acad Sci USA 89:2950-4).

Inhibition by MCVCC of chemokine-induced chemotaxis of neutrophils, obtained by dextran sedimentation and hypotonic lysis (Clark *et al.*, 1993 Curr. Protocols Immunol. 2, 7.23.1-7.23.4) of random donor buffy coats, was analyzed in a 48 well microwell Boyden chamber (Neuroprobe) fitted with a 3 μm pore PVP-free polycarbonate membrane and a 30 minute incubation at 37°C. Cells and chemokines were diluted into calcium- and magnesium-free HBSS (GibcoBRL) containing 0.1% bovine serum albumin.

Inhibition by MCVCC of chemokine-induced chemotaxis of elutriated lymphocytes, isolated using Ficoll Hypaque gradient centrifugation, was analyzed in a 48 well microwell Boyden chamber (Neuroprobe) with mouse type IV collagen coated PVP-free polycarbonate filters (pore size 5 μm) and a two hour incubation at 37°C.

The inhibitory effect of MCVCC on the ability of chemokines to induce alterations in intracellular calcium concentration was also demonstrated. Cells were loaded with Fura-2 (Molecular Probes) using methods well known to one of skill in the art (Leonard, E.J., et al., 1995, *supra*) and fluorescence was monitored in a PTI spectrofluorometer. Results were expressed as the ratio of fluorescent emission at 510 nm from repetitive, sequential, excitations at 340 nm and 380 nm.

The ability of MCVCC to compete with chemokines for binding to whole cells was demonstrated. One million cells bearing the receptor(s) of interest in binding buffer (RPMI + 0.5% BSA + 25 mM Hepes, pH 7.4) were dispensed to triplicate tubes containing 0.1 nM ^{125}I-labeled chemokine (2200 Ci/mmol) and either unlabeled chemokine or unlabeled MCVCC. Binding was allowed to occur at 4°C with gentle agitation for 2 hours. To separate unbound from bound radioactivity, the cells were first centrifuged for 5 minutes at low speed in a microfuge. The pellet was then resuspended in 200 μl of binding buffer and layered over a 1 ml 10% sucrose/phosphate buffered saline cushion. After centrifugation at 10,00 rpm in a microfuge, the supernatant was aspirated and the cell associated radioactivity was quantitated in a gamma counter.

The ability of MCVCC to inhibit fusion mediated by interaction of the HIV-1 envelope protein with membrane bound chemokine receptors was demonstrated. The procedure of Nussbaum et al. (Feng, Y., et al., 1996, "HIV-1 entry cofactor:function cDNA cloning of a seven-transmembrane G protein-coupled receptor," Science 272:872-877; Nussbaum, O., et al., 1994, "Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantifying cell fusion-dependent reporter gene activation," J. Virol. 68:5411-22) was used to measure fusion

inhibitory activity. This procedure has been shown to correlate with inhibition of HIV-1 infectivity.

EXAMPLE 3:

INHIBITION BY MCVCC OF THE CHEMOTAXIS OF MONOCYTES INDUCED BY CC CHEMOKINES

Purified MCVCC did not induce the chemotaxis of monocytes indicating that it did not have chemokine agonist activity. The ability of MCVCC to function as a chemokine antagonist was tested. Because of the structural similarity of MCVCC to CC chemokines, initial studies evaluated the ability of the protein to antagonize the function of CC chemokines. In each chemotaxis inhibition experiment, the concentration of chemokine used was that which elicited half maximal to full maximal chemotactic effect as determined in a parallel assay. MCVCC inhibited monocyte chemotaxis to the CC chemokines RANTES, MIP-1α, MCP-1, MCP-3 and I-309 (Fig. 2). A ten- to fifteenfold molar excess of MCVCC inhibited 70-85% of the monocyte chemotaxis that occurred with chemokine alone. The effect of MCVCC was specific: heat treatment of MCVCC for 15 minutes at 96°C abrogated its chemotactic inhibitory effect and excess of a CXC chemokine (IL-8) had no effect on monocyte chemotaxis to a CC chemokine (Fig. 2). In addition, MCVCC was specific for chemokines, since the protein did not antagonize the activity of the classical chemoattractant fMLP (Fig 2).

EXAMPLE 4:

MCVCC IS AN ANTAGONIST OF CXC CHEMOKINES

Because a broad spectrum inflammatory agent should have activity against both major classes of chemokines, the ability of MCVCC to inhibit the activity of the CXC chemokine SDF-1 was determined. In a monocyte chemotaxis assay, MCVCC inhibited the activity of 50 nM SDF-1 by 70% (Fig. 2).

A broad spectrum inflammatory agent should inhibit chemokine-induced chemotaxis of lymphocytes and neutrophils as well as monocytes. SDF-1 is the most potent known chemoattractant of lymphocytes. A ten- to fifteenfold molar excess of MCVCC over SDF-1 inhibited SDF-1 induced lymphocyte chemotaxis by 70-80% (Fig. 2). The specificity of the effect was demonstrated using heat inactivated MCVCC or an ineffective β chemokine as a control.

A ten- to fifteenfold molar excess of MCVCC to the CXC chemokine IL-8 inhibited IL-8 induced chemotaxis of neutrophils by 70 to 80% (Fig. 2). The specificity of the effect was demonstrated using heat inactivated MCVCC or an ineffective β chemokine as a control.

EXAMPLE 5:
INHIBITION BY MCVCC OF CALCIUM FLUX INDUCED BY CC AND CXC CHEMOKINES

The release of intracellular calcium may be induced by the binding of a chemokine to a cellular receptor. A calcium flux did not occur upon incubation of primary monocytes (Fig. 3) or primary PBMC with MCVCC indicating the absence of agonist activity. Nevertheless, MCVCC exhibited a dose-dependent ability to inhibit calcium fluxes to MCP-1 in primary monocytes and CCR2b transfected HEK293 cells (Fig. 3). MCVCC also inhibited the calcium flux (data not shown) to a submaximal stimulation with 5 nM MIP-1α or 5 nM RANTES in CCR1 and CCR5 transfected CD4 HOS cells obtained from the AIDS repository (deposited by N. Landau). Half maximal calcium fluxes to 0.5 nM I-309, a chemokine which appears to be specific for CCR8, were inhibited by pre treatment with MCVCC (Fig. 3) in CCR8 transfected murine preB cells (a kind gift of L.Tiffany and P. Murphy).

MCVCC also exhibited ability to inhibit calcium fluxes to SDF-1α. Pretreatment with MCVCC of a murine pre-B cell line with CXCR4 receptors inhibited the calcium flux induced by 0.1 nM SDF-1α.

EXAMPLE 6:

INHIBITION BY MCVCC OF THE BINDING OF CC CHEMOKINES TO CELLS

MCVCC competitively inhibited binding of radiolabeled MCP-1 to CCR2b HEK293 cells (Fig. 4A), consistent with the ability of MCVCC to inhibit chemotaxis of monocytes to MCP-1 and to MCP-3, and to inhibit calcium fluxes to primary monocytes and to CCR2b transfected HEK293 cells. With an IC_{50} equivalent to that of MCP-1, MCVCC inhibited binding of MCP-1 to CCR2b. These results indicate that MCVCC competitively inhibits binding of MCP-1 to the chemokine receptor CCR2b.

A second experiment demonstrating the ability of MCVCC (MC148P) to compete for I-309 binding to CCR8 transfected murine pre B cell line was modified from the above protocol. Binding occured at 26°C for one hour in Hanks buffered salt solution (HBSS) containing 1% BSA and 0.1% azide in a 100 ul volume. Separation from bound from free radioactively labelled I-309 was accomplished busing a 10-fold excess of binding buffer containing 0.5M NaCl. Cell bound radioactivity was pelleted at 14,000 rpm in a microifuge for 5 minutes. After aspiration of the supernatant, pellet-associated radioactivity was counted in a gamma counter. The concentration of labelled I-309 and number of cells was the same as previously described. Figure 4B shows a graph illustrating that MCVCC competes with I-309 for binding to CCR8 transfected murine preB cells. The IC_{50} of I-309 and MCVCC are similar.

EXAMPLE 7:

INHIBITION BY MCVCC OF FUSION INDUCED BY THE HIV-1 ENVELOPE PROTEIN

A plasmid encoding the HIV-1 strain 89.6 envelope was transfected into HeLa cells using DOTAP reagent (Boehringer Mannheim). Subsequently, HeLa cells were infected with recombinant vaccinia virus vCB-21R (Alkhatib, G., et al., 1996, "Cell type-specific fusion cofactors determine human immunodeficiency virus type 1 tropism for T-cell lines versus primary macrophages," J. Virol. 70:5487-94) which encodes *lacZ* regulated by a bacteriophage T7 promoter. Similarly, 3T3 cells were transfected with plasmid pSC59 (Chakrabarti, S., et al., 1997, "Compact, synthetic, vaccinia virus early/late promoter for protein expression," BioTechniques 21:1904-7) as a control or with pSC59 encoding CCR5. After 4 hours, the 3T3 cells were infected with recombinant vaccinia virus vCB3 expressing CD4 (Broder, C.C., et al., 1993, "The block to HIV-1 envelope glycoprotein-mediated fusion in animal cells expressing human CD4 can be overcome by a human cell component(s)," Virol. 193:483-491) and vTF7-3 encoding the bacteriophage T7 RNA polymerase. After separate incubation of the HeLa and 3T3 cells overnight at 32°C, 100,000 HeLa cells were mixed with 100,000 3T3 cells in the presence or absence of MIP-1α, MCVCC, or IL-8. After 2 hours at 37°C, NP-40 was added to 0.5%; β-galactosidase activity was assayed using 8 mM chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim) as the substrate. MCVCC inhibited the fusion of murine 3T3 cells expressing CCR5 and CD4 with HeLa cells expressing HIV-1 strain 89.6 envelope protein. The inhibitory effect of MCVCC was more potent than that of MIP-1α in the assay (Fig 4). Whereas 10 nM MIP-1α inhibited fusion by <10%, 10 nM MCVCC inhibited fusion by almost 50%. Both MIP-1α and MCVCC had similar effects at 100 nM. This, along with the chemotaxis and calcium flux data, implies that MCVCC interacts with the CCR5 receptor and indicates that MCVCC may be a potent inhibitor of HIV entry.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for treating a chemokine related immunopathological disorder in a subject, comprising administering to the subject a therapeutically effective amount of an anti-inflammatory protein encoded by molluscum contagiosum virus (MCV) from about nucleotide 166,992 to nucleotide 167,303, or a biologically active fragment thereof.

2. The method of claim 1, wherein the chemokine is a class α (CXC) or a class β (CC) chemokine.

3. The method of claim 1, wherein the anti-inflammatory protein has an amino acid sequence as set forth in SEQ ID NO: 2.

4. The method of claim 2, wherein the chemokine is a class α (CXC) chemokine.

5. The method of claim 4, wherein the chemokine is selected from the group consisting of CTAP-III, gro/MGSA, ENA-78, interleukin-8, PF-4, SDF-1, and NAP-2.

6. The method of claim 2, wherein the chemokine is a class β (CC) chemokine.

7. The method of claim 6, wherein the chemokine is selected from the group consisting of MCP-1, RANTES, MIP-1α, MIP-1β, MCP-3, and I-309.

8. The method of claim 1, wherein the immunopathological disorder is selected from the group consisting of microbial infection, malignancy and metastasis, asthma, coronary restenosis, autoimmune diseases, cirrhosis, endotoxemia, rheumatoid arthritis, atherosclerosis, psoriasis, inflammatory bowel disease, interstitial cystitis and reperfusion injury.

9. The method of claim 1, further including administering an antibiotic or antiviral agent to the subject.

10. The method of claim 1, wherein the administering of anti-inflammatory protein is selected from the group consisting of subcutaneous, intravenous, intraarterial, intramuscular, intrarectal, local and transdermal.

11. The method of claim 1, wherein the anti-inflammatory protein is introduced to the subject using a carrier.

12. The method of claim 11, wherein the carrier is a vector.

13. The method of claim 1, wherein the administering is *ex vivo*.

14. The method of claim 1, wherein the administering is *in vivo*.

15. A method of treating a subject having or at risk of having an HIV infection or disorder, comprising administering to the subject, a therapeutically effective amount of an anti-inflammatory protein encoded by molluscum contagiosum virus (MCV) from about nucleotide 166,992 to nucleotide 167,303, or a biologically active fragment thereof.

16. The method of claim 15, wherein the anti-inflammatory protein has an amino acid sequence as set forth in SEQ ID NO: 2.

17. The method of claim 15, wherein the subject is suffering from AIDS or ARC.

18. The method of claim 15, wherein the polypeptide is formulated in a pharmaceutically acceptable carrier.

19. The method of claim 15, further comprising administering an antiviral agent to the subject.

20. A pharmaceutical composition comprising at least one dose of an immunotherapeutically effective amount of an anti-inflammatory protein encoded by molluscum contagiosum virus (MCV) from about nucleotide 166,992 to nucleotide 167,303, or a biologically active fragment thereof, in a pharmaceutically acceptable carrier.

21. The pharmaceutical composition of claim 20, wherein the anti-inflammatory protein has an amino acid sequence as set forth in SEQ ID NO: 2.

22. The pharmaceutical composition of claim 20, wherein the anti-inflammatory protein is a class α or a class β chemokine antagonist.

23. The pharmaceutical composition of claim 22, wherein the chemokine is selected from the group consisting of CTAP-III, gro/MGSA, ENA-78, MCP-1, interleukin-8, RANTES, MIP-1α, MIP-1β, PF-4, I-309, SDF-1 and NAP-2.

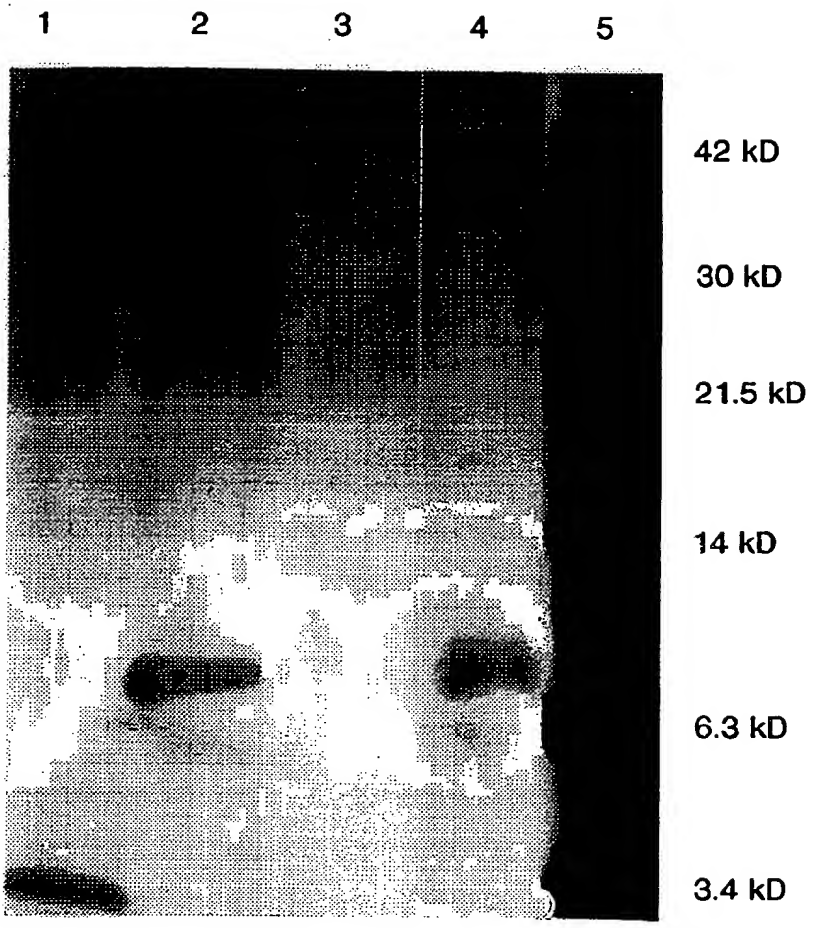

FIG. 1

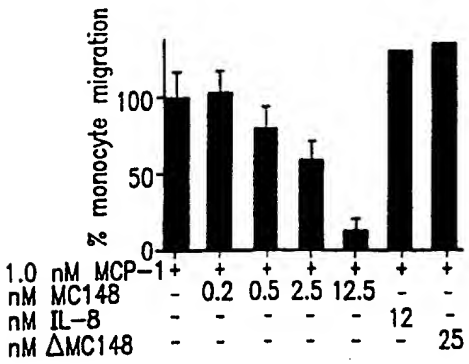

FIG. 2A

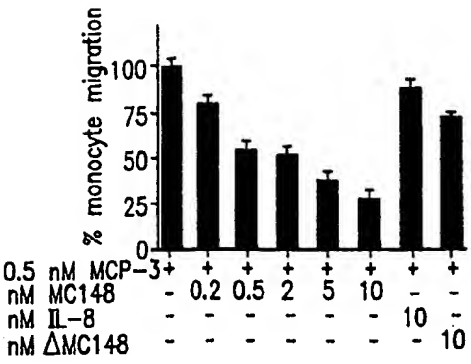

FIG. 2B

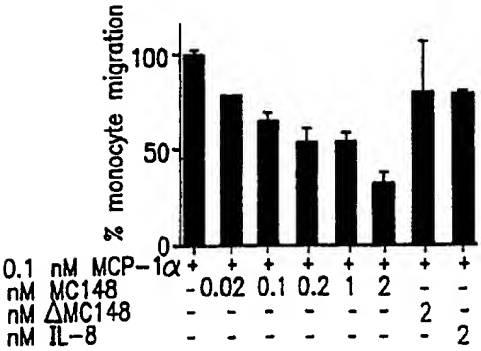

FIG. 2C

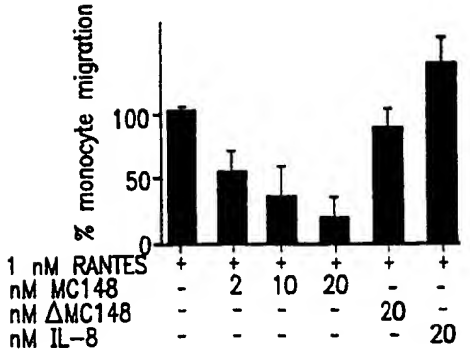

FIG. 2D

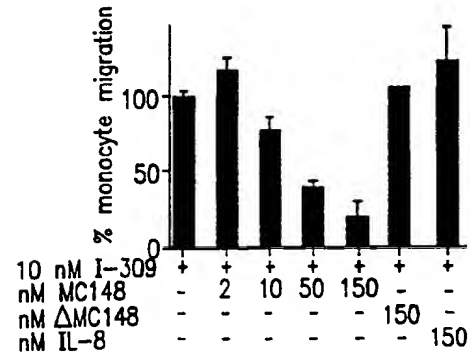

FIG. 2E

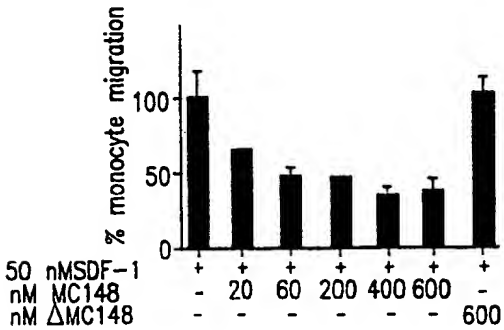

FIG. 2F

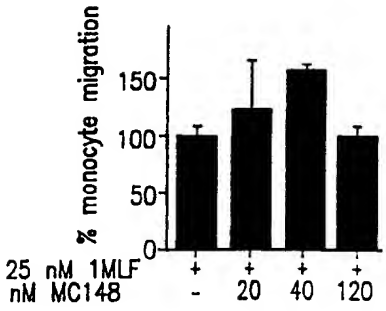

FIG. 2G

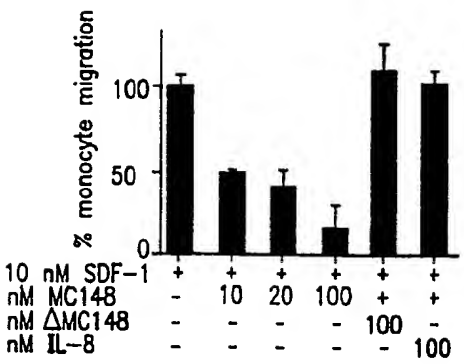

FIG. 2H

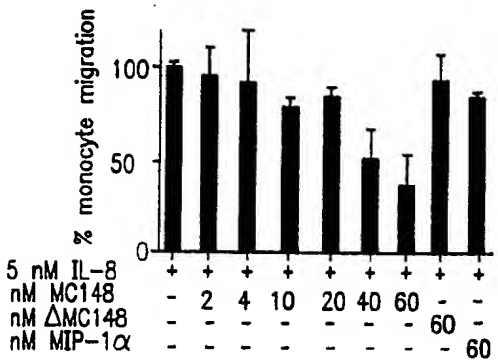

FIG. 2I

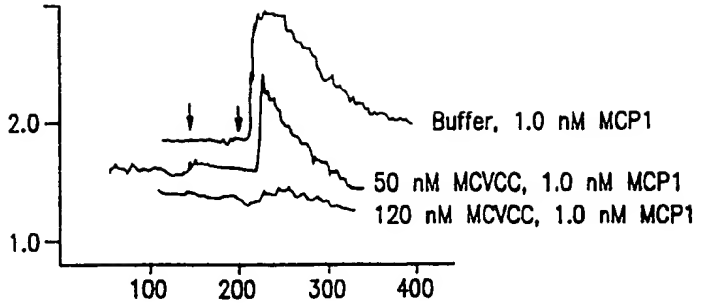

FIG. 3A

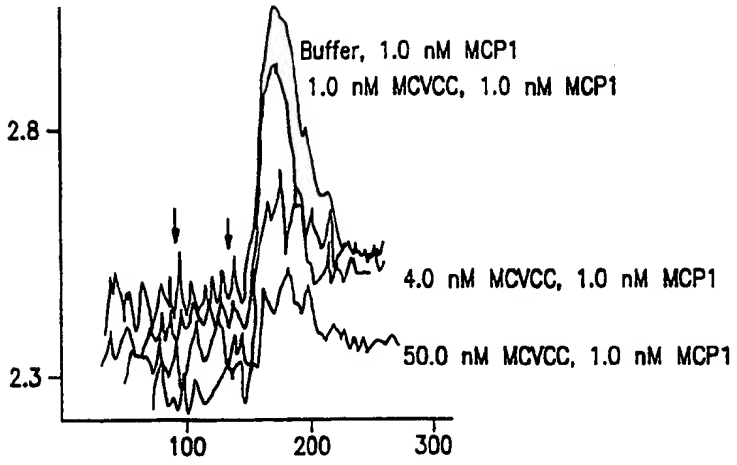

FIG. 3B

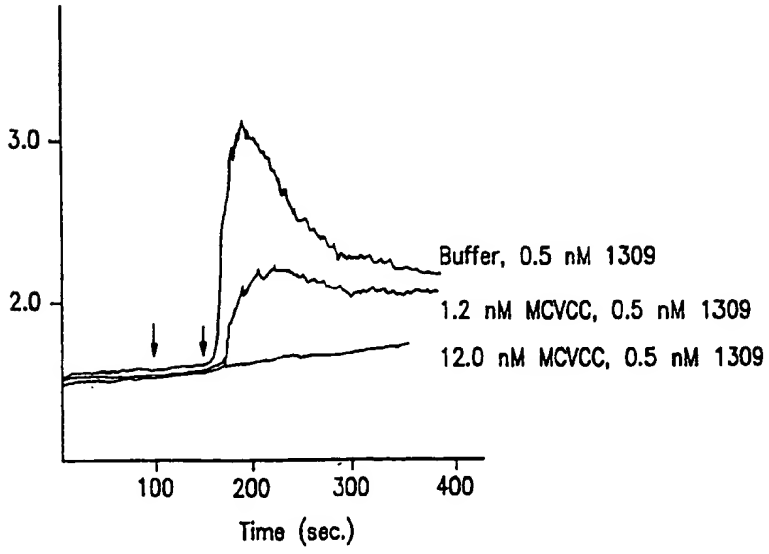

FIG. 3C

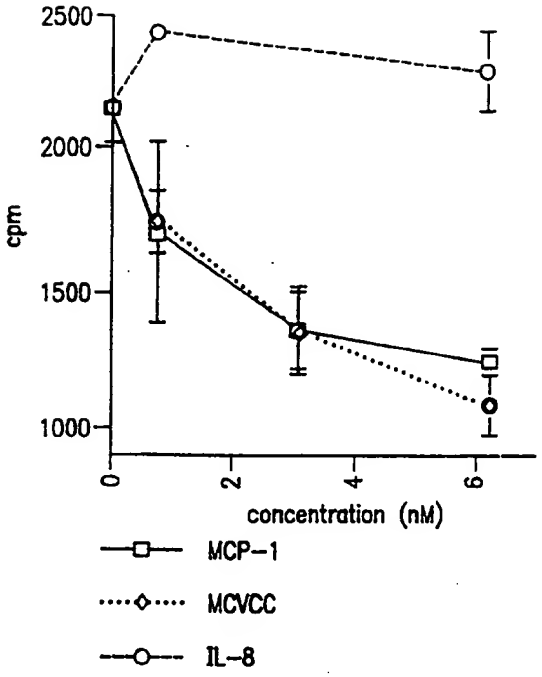
2500
2000
1500
1000
cpm
0
2
4
6
concentration (nM)
MCP-1
MCVCC
IL-8

FIG. 4A

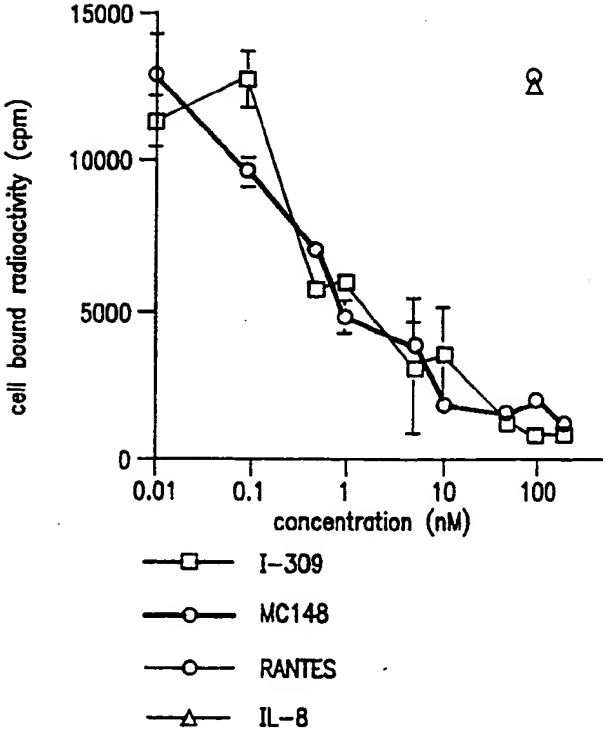
15000
10000
5000
0
cell bound radioactivity (cpm)
0.01
0.1
1
10
100
concentration (nM)
I-309
MC148
RANTES
IL-8

FIG. 4B

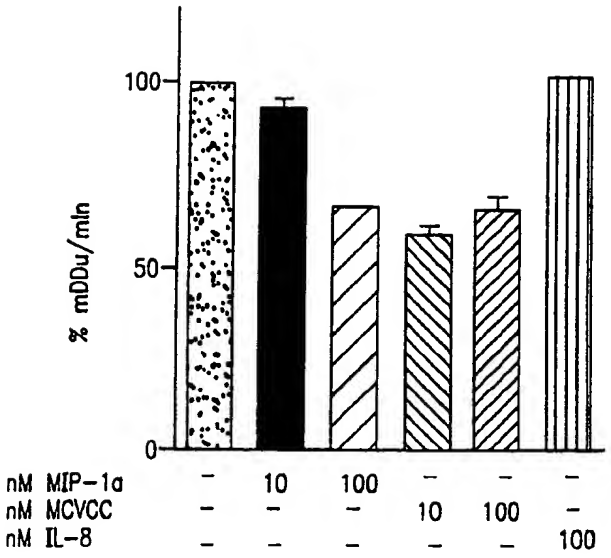

FIG. 4C